Effects on Endotoxin Pathogenicity in Pigs with Acute Septicemia of *Haemophilus parasuis* Infection

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(Received 28 November 1996/Accepted 27 February 1997)

ABSTRACT. Changes of endotoxin in plasma, and the response of the coagulation system and blood cells in septicemia of *Haemophilus parasuis* infection were examined by inoculation with *H. parasuis* in specific pathogen-free (SPF) pigs. Eight pigs were inoculated intratracheally with 10^5, 10^6 and 10^7 colony formation units (CFU) of the strain Nagasaki (serovar 5). All pigs died 28 to 42 hr after inoculation. Haematologically, severe leukopenia occurred 24 hr post inoculation (hpi) until death. Glucose concentration decreased from 24 hpi to death. In the coagulation system, decrease of platelet counts, prolongation of prothrombin time, activated partial thromboplastin time, and increase of fibrinogen-fibrin degradation products were observed in all inoculated pigs. Endotoxin was detected in the plasma of all the inoculated pigs from 16 hpi to death, and its concentration rose dramatically just before death. *H. parasuis* was re-isolated from the blood of all inoculated pigs from 16 hpi to death, and also from almost all organs and body fluids of the pigs. The pigs had microthrombi in the kidney, liver and lungs, and many also had pneumonia, meningitis and serositis. *H. parasuis* antigen was detected in the lesions by the immunoperoxidase technique. The results indicated that disseminated intravascular coagulation (DIC) and endotoxin shock involved aggravation of clinical signs and death on the pigs induced to septicemia of *H. parasuis*. — KEY WORDS: disseminated intravascular coagulation, endotoxin, *Haemophilus parasuis*, Glasser's disease, swine.

J. Vet. Med. Sci. 59(6): 451–455, 1997

There are two types of *Haemophilus parasuis* infection. The first type is Glasser's disease, and the other is the septic type [13, 16, 18]. *H. parasuis* infection occasionally shows very serious enzootic characteristics with a very high morbidity [12, 16] and acute septicemia type in specific pathogen-free (SPF) herds. Thrombosis has been observed on the renal glomerulus and the other organs in these acute septicemic cases [1, 16, 18]. It is known that the effects of endotoxin are involved in formation of microthrombi in Gram-negative bacterial infection [9]. In a previous report, we detected endotoxin in the blood of pigs with Glasser's disease and acute septicemia induced by *H. parasuis*, and indicated that endotoxins might play an important role in the pathogenesis of septicemic lesions of the *H. parasuis* infection [2]. However, the relationship between endotoxin and thrombosis has not been clear in *H. parasuis* infection in pigs. The present study was undertaken to confirm the correlation of endotoxin and thrombosis by the periodic examination of endotoxin and the coagulation system in the blood of SPF pigs intratracheally inoculated with *H. parasuis* (serovar 5).

MATERIALS AND METHODS

Animal: Ten Large White pigs, 9 weeks olds and SPF pigs, were used in the experiment. They were negative for the isolation of *H. parasuis* from the nasal cavities and for complement-fixing (CF) antibodies against *H. parasuis*.

Bacteria: *H. parasuis* reference strain Nagasaki (serovar 5) were used as inocula. Strain Nagasaki was isolated from meninges of a septicemic pig with meningitis [8]. Bacterial inocula were prepared from 18 hr cultures in brain heart infusion agar (BBL) supplemented with 200 mg of β-nicotinamide adenine dinucleotide (Sigma) per liter (BHI agar). The preparation of inocula followed the method described previously [2]. Briefly, the cultures grown on the BHI agar plate at 37°C for 18 hr were harvested and suspended in 0.01 M phosphate-buffered saline, (PBS) pH 7.2. After washing twice with PBS, each bacterial suspension was diluted with PBS to provide viability of 3.2×10^4, 3.2×10^5 and 3.2×10^6 colony-forming units (CFU) per 1 m*l*.

Experimental procedure: Before inoculation of *H. parasuis* or PBS, all pigs were sedated with azaperone (8 mg/kg body weight, intramuscularly (IM)), and ketamine (10 mg/kg, IM), and atropine (0.05 mg/kg, IM), then anesthetized with thiopental sodium (10 mg/kg, intravenously), and restrained with head in a vertical position. A catheter (about 1 cm in diameter) was advanced through the larynx deep into the bronchi, and 5 m*l* of bacterial suspension was slowly administered. The catheter was then quickly withdrawn, and the throat was massaged to prevent coughing. Control pigs were inoculated in the same way with 5 m*l* of sterile PBS. Eight pigs were intratracheally inoculated with *H. parasuis*; two pigs (Nos. 1 and 2) were inoculated intratracheally with 1.6×10^7 CFU of *H. parasuis*, three pigs (Nos. 3, 4, and 5) with 1.6×10^6, and another three pigs (Nos. 6, 7, and 8) with 1.6×10^5 CFU. The remaining two pigs, as controls, were inoculated with PBS only, and housed separately from the inoculated pigs. After inoculation, all pigs were observed for clinical signs of disease during the experiment.

Sampling of blood: After inoculation, clinical examinations were made, and rectal temperature was recorded. Blood samples were obtained from the anterior vena cava in serum tubes pre-inoculation and on 8, 16, 24, 32, and 40 hr post inoculation (hpi).

Hematological tests and detection of endotoxin: The counts of white blood cells (WBC) and plates were carried out by the standard procedures. In the blood coagulation test, one stage prothrombin (PT) and activated partial thromboplastin time (APTT) were measured with commercial reagents for human blood coagulation tests (Dade Diagnoatics, Inc., Delaware Parkway, Miami, U.S.A.) using the Micro-Coagulometer (Greiner Electronics Co., Switzerland). Fibrinogen-fibrin degradation product (FDP) was measured by Latex agglutination method with commercial reagents (Kokusai Shiyaku Co., Tokyo). Biochemical determinations were carried out with DRY-CHEM systems (Fuji Film Co., Tokyo). Fibrinogen was measured by thrombin time assay method using commercial reagents (Dade). Sialic acid (SA) was measured by the enzyme method using a commercial kit (Kyokuto Pharmaceutical Co., Tokyo). Mucoprotein (MP) was measured by the Coomasie Brilliant Blue G-25 method using commercial reagents (Otsuka Assay Lab., Osaka). Endotoxin concentration in plasma was determined by an automated turbidimetric time assay instrument (Toxinometer ET-201, Wako) as previously described [15, 20].

Bacterial examination: Samples of blood and body fluids (peritoneal, pleural, synovial and cerebrospinal), trachea, brain, and viscera were inoculated onto BHI agars. The cultures were incubated at 37°C for 24 hr in an atmosphere containing 5% carbon dioxide, and the confirmation of isolates was performed as in the previous report [6].

Pathological examination: The dead pigs were necropsied as soon as possible. The control pigs were euthanatized at the end of the experiment, at 48 hpi. Specimens of each organ were fixed in 20% buffered formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E), and phosphotungstic acid-hematoxylin (PTAH). The bacterial antigen of *H. parasuis* was demonstrated by the avidin-biotin complex immunoperoxidase (ABC) method, using a Vectastain ABC kit (Vector Lab., U.S.A.) according to the method described previously [2]. Briefly, rabbit anti-Nagasaki of *H. parasuis* preimmune sera were used as primary antibodies at a dilution of 1:8,192. The slides were successively incubated with ABC, and peroxidase activity was demonstrated by treating them with 3,3'-diaminobenzidine tetra-hydrochloride. The slides were then counterstained with methyl green.

RESULTS

Clinical observation: All pigs inoculated with organisms showed pyrexia and depression from 16 hpi, and respiratory distress, cyanosis and recumbency from 24 hpi. Four pigs (Nos. 1, 4, 5, and 8) showed lameness and/or convulsion. They died 28 to 42 hpi (Table 1). Body temperature rose over 40°C in all pigs at 16 or 24 hpi, but it fell below that level at death in four pigs (Nos. 1, 2, 5, and 6). The control pigs exhibited no clinical signs during the observation period.

Bacterial examination: Results of re-isolation of *H. parasuis* from the blood and the dead pigs are shown in Table 1. *H. parasuis* were re-isolated from the blood of two pigs (Nos. 1 and 5) at 8 hpi, and the remaining five pigs at 16 hpi, then from the blood of all infected pigs until death. Numbers of isolated organisms ranged from 10^2 to 10^5 CFU per m*l* of blood (Table 1). *H. parasuis* was recovered from tracheae, brain, liver, spleen, kidney, lung, heart, and body fluids of the affected pigs. The control pigs were negative of *H. parasuis*.

Haematological and biochemical measurements: WBC counts of the affected pigs increased from 8 hpi, but significant ($P<0.05$) leukopenia occurred at 24 to 40 hpi (Table 2). Platelet counts of the infected pigs decreased continuously from 24 hpi until death (Table 2). Glucose concentration decreased at 24 hpi, then continually decreased further until death. Total serum protein, albumin concentration, GOT and BUN activities were within the

Table 1. Results of clinical and bacterial examination of pigs inoculated with *H. parasuis*

Pig No.	Infecting dose	Result of clinical examination		Isolation of *H. parasuis* from blood						Isolation of[a] *H. parasuis*
		Clinical sign	Time of death (hr)	0*	8**	16	24	32	40 hr	from dead pigs
1	10^7	+[b]	38	–	1+[c]	3+[e]	3+	3+		+
2	10^7	+	34	–	–	2+	3+	3+		+
3	10^6	+	42	–	–	2+	2+	3+	3+	+
4	10^6	+	38	–	–	3+	3+	3+		+
5	10^6	+	28	–	2+[d]	3+	3+			+
6	10^5	+	30	–	–	3+	3+			+
7	10^5	+	34	–	–	3+	3+	3+		+
8	10^5	+	38	–	–	2+	3+	3+		+

a) The organism was isolated from the liver, spleen, kidney, lung, heart, brain, trachea, and body fluids (synovial and cavity). b) +: Positive; –: Negative; c) 1+: 1–10^2 colony formation unit (CFU)/m*l*; d) 10^2–10^4 CFU/m*l*; e) 10^4–10^5 CFU/m*l*; *0: Before inoculation; **: Hr after inoculation.

Table 2. Changes in blood cells, glucose, sialic acid, and mucoprotein after inoculation of *H. parasuis*

Pig	Examination item	Time (hr) after inoculation					
		0 (n=8)	8 (n=8)	16 (n=8)	24 (n=8)	32 (n=6)	40 (n=1)
Inoculated Pigs	WBC[a] ($\times 10^3$)	11.4 ± 2.5	15.4 ± 4.9*	13.8 ± 9.8	5.5 ± 3.5**	3.6 ± 4.2**	0.8
	Glucose (mg/m*l*)	135 ± 17	ND	ND	70 ± 21**	61 ± 15**	54
	Sialic acid (mg/m*l*)	68.5 ± 11.2	73.7 ± 20.1	79.3 ± 13.0	87.5 ± 17.2**	99.4 ± 18.0**	102.9
	Mucoprotein (mg/m*l*)	201 ± 16	241 ± 39	263 ± 35**	296 ± 51**	336 ± 73**	340
	Platelet counts[b]	47.0 ± 10	47.9 ± 11.2	43.7 ± 12.8	25.9 ± 4.4**	18.3 ± 11.2**	11.2
	Fibrinogen (mg/m*l*)	155 ± 5	151 ± 16	203 ± 45**	308 ± 55**	341 ± 32**	310

a) White blood cell. b) $\times 10^4$. Data are given as mean ± SD. Statistically significant (*=P<0.05, **=P<0.01) difference between that value indicated and the mean value before inoculation.

Table 3. Changes in coagulation system of pigs inoculated with organism

Pig No.	Item	Time (hr) after inoculation					
		0	8	16	24	32	40
1	PT[a]	15.5[d]	13.7	29.0	21.2	36.4	
	APTT[b]	21.2[e]	13.0	70.6	42.4	69.0	
	FDP[c]	<2.5[f]	<2.5	2.5	2.5	5.0	
2	PT	15.0	13.9	17.9	17.6	21.4	
	APTT	14.4[e]	12.0	34.0	27.5	NT[g]	
	FDP	<2.5	<2.5	2.5	2.5	5.0	
3	PT	18.9	16.6	19.1	18.5	18.4	27.4
	APTT	22.4	17.5	39.5	21.2	26.6	52.6
	FDP	<2.5	<2.5	<2.5	<2.5	2.5	>10.0
4	PT	17.1	14.9	19.8	18.6	21.2	
	APTT	20.0	15.7	35.7	41.8	48.5	
	FDP	<2.5	<2.5	2.5	2.5	5.0	
5	PT	16.7	15.7	22.9	41.0		
	APTT	20.4	17.4	36.0	103.5		
	FDP	<2.5	<2.5	<2.5	2.5		
6	PT	17.2	14.8	22.2	20.7		
	APTT	21.8	15.8	51.1	41.3		
	FDP	<2.5	<2.5	2.5	5.0		
7	PT	16.1	15.1	16.3	17.7	20.3	
	APTT	28.3	23.0	24.7	23.7	68.6	
	FDP	<2.5	<2.5	<2.5	2.5	10.0	
8	PT	18.2	17.2	18.7	21.7	23.7	
	APTT	23.7	26.3	20.0	34.3	48.6	
	FDP	<2.5	<2.5	<2.5	2.5	5.0	

a) Prothrombin time. b) Activated partial thromboplastin. c) FDP=Fibrinogen-fibrin degradation product. d) seconds. e) seconds. f) μg/m*l*. g) NT=not tested.

normal range during the experiment. The concentration of sialic acids and mucoproteins in the serum of the affected pigs showed an increase from 8 or 16 hpi, and then a continuous increase until death (Table 2).

Blood coagulation tests: Fibrinogen of the infected pigs showed a significant (P<0.01) increase from 16 hpi until death (Table 3). PT and APTT in the infected pigs shortened slightly at 8 hpi, and then prolonged slowly from 16 hpi in all pigs. APTT prolonged considerably just before death in some pigs. FDP became positive at 16, 24 or 32 hpi in all the infected pigs, and its value increased after that.

Table 4. Detection of endotoxin from blood of pigs inoculated with organism

Pig No.	Time (hr) after inoculation						Death time (hr)
	0	8	16	24	32	40	
1	–[a]	–	0.45[b]	0.67	20.4		38
2	–	–	0.75	37.3	438.0		34
3	–	–	–	0.07	0.07	120.0	42
4	–	–	0.45	0.59	39.2		38
5	–	–	16.1	662.0			28
6	–	–	1.04	21.45			30
7	–	–	0.04	33.6	515.5		34
8	–	–	0.2	0.31	12.11		38

a) –: 0.02 endotoxin unit/m*l*. b) endotoxin unit/m*l*.

Endotoxin measurements: Endotoxin was detected in the plasma of all pigs infected with *H. parasuis* from 16 or 24 hpi, its concentration rising dramatically just before death. The endotoxin levels in four pigs (Nos. 2, 3, 5, and 7) were especially high (120.0 to 662.0 EU per m*l*) at this time.

Pathological and immunopathological examination: The necropsy findings consisted principally of congestion and edema in many organs. Seven pigs except for pig No. 2 had fibrinous serositis (peritonitis, pericarditis, and pleuritis). Four pigs (Nos. 1–4) had focal consolidation in the lung. The histopathological and immunopathological findings, in brief, show that all pigs had septicemic lesions, and fibrinous microthrombi in the kidney, lung, and liver. Four pigs (Nos. 1, 4, 5 and 8) had mild meningitis. Focal bronchopneumoniae were evident in four pigs (Nos. 3, 5, 6, and 8) and mild pleuropneumonia in 3 pigs (Nos. 1, 2, and 4). No lesions were observed in the controls. *H. parasuis* antigen was found mainly in the small vessels of almost all organs, and in the cytoplasma of infiltrating cells in the serositis, meningitis, and pneumonia by ABCIT in all infected pigs.

DISCUSSION

Our previous report indicated that nine of ten pigs inoculated intranasally with 10^6 to 10^{10} CFU of *H. parasuis* strain Nagasaki died with septicemia or meningitis from 2 to 6 days post inoculation [2]. Although a lower dose of organisms than in the previous experiment was inoculated into the trachea in the present study, all pigs showed septicemia and died within two days; they had severer clinical signs and lesions. The difference in the pathogenicity of the organisms might be attributed to the inoculation route site, because an organism inoculated intratracheally circumvents the mucousal immunity of the upper respiratory tract, and causes systemic infection more easily. This result indicates that the intratracheal infection method may be unsuitable for production of acute septicemia in *H. parasuis* infection.

The organisms were isolated from slight pneumonic lesions in natural infection [5, 13]. However, pneumonia would not be observed in spontaneous Glasser's disease [3, 14] and had not been induced by intranasal inoculation in SPF pigs in our previous report [2]. This may suggest that *H. parasuis* can not easily produce pneumonia in natural conditions. A large dose of *H. parasuis* was required to induce pneumonia by intratracheal inoculation [18]. In the present study, pleuropneumonia or bronchopneumonia was observed in most pigs exposed to *H. parasuis*. This indicates that pneumonia might be produced when *H. parasuis* can invade the lung, or the defence mechanisms of the respiratory tract were deflected by some other cause.

Endotoxin is a component of the outer membrane of gram-negative bacteria, and is released with bacterial lysis [7]. DIC is sometimes induced and endotoxin detected in animals infected with gram-negative bacteria [4]. There are some reports that thrombosis in the kidney, lung and brain was observed in acute septicemia of natural *H. parasuis* infection. However, these reports did not confirm DIC in the animals [18, 20]. In our previous report [2], DIC and high concentrations of endotoxin were detected in the blood of acute septicemic pigs. However, we did not examine the correlation between the development of DIC and endotoxin concentration in the blood in acute septicemia of *H. parasuis* infection. In the present study, clinical signs and septicemia were demonstrated in all pigs after 16 hpi, and endotoxin and a disorder of the coagulation system were detected simultaneously. The prolonging of PT and APTT, the decrease of platelets, and the increase of FDP suggested the development of DIC. Microthrombosis was observed in the kidney, liver, and lungs of the dead pigs, histopathologically. Therefore it was considered that DIC was induced from the onset of acute septicemia, and aggravated the clinical signs such as dyspnea and cyanosis.

It is well known that animals injected intravenously with endotoxin from *E. coli* suffer endotoxin shock and show circulatory failures such as a drop in blood pressure, leukopenia and hypoglycemia [11, 19]. Blood pressure was not examined in this study, but all the infected pigs were regarded as sustaining endotoxin shock according to the clinical signs, leukopenia and hypoglycemia. Thus, it has been assumed that pigs with septicemia of *H. parasuis* would suffer endotoxin shock, then DIC, and die within a short time. Most biological activities of endotoxin can be attributed to mediators which macrophages have produced through stimulation by endotoxin [10, 12]. Tumor necrosis factor (TNF) in the mediators is most related with endotoxin shock and thrombosis lesions [10, 12]. TNF production was not examined in this study, but it has been assumed that TNF would be induced in the septicemia of *H. parasuis* infection.

There have been few reports about the change of endotoxin concentrations in the blood of animals infected with gram-negative bacteria. In the present study, the concentration of endotoxin in plasma showed a slow increase at first, and then rose dramatically just before death in some pigs. This finding indicates that DIC and shock increased, but then fell at death in some pigs. Therefore, it is considered that treatment for endotoxin shock and DIC is important, in addition to the administration of antibiotics in acute septicemia.

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